

**TUBERCULOUS MENINGITIS: EVALUATION OF POLYMERASE CHAIN REACTION AS A DIAGNOSTIC TOOL – A PILOT STUDY***MENINGITE TUBERCULOSA: AVALIAÇÃO DA REAÇÃO EM CADEIA DA POLIMERASE COMO FERRAMENTA DIAGNÓSTICA – UM ESTUDO PILOTO*

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**ABSTRACT**

Meningitis is a severe and potentially fatal form of tuberculosis. The diagnostic workup involves detection of acid-fast bacilli in the cerebrospinal fluid by microscopy or culture. However the difficulty in detecting the organism poses a challenge to diagnosis. Use of polymerase chain reaction (PCR) in the diagnostic approach to *Mycobacterium tuberculosis* (MTB) meningitis has been reported as a fast and accurate method, with several commercial kits available. As an alternative, some institutions have been developing inexpensive in-house assays. In our institution, we use an in-house PCR for tuberculosis. The performance of our PCR for the diagnosis of MTB meningitis was analyzed in 148 consecutive patients, using MTB culture as the gold standard. Sensitivity of cerebrospinal fluid PCR for the diagnosis of MTB meningitis was 50%, specificity was 98.6%, and concordance with culture was 96% (kappa = 0.52). The performance of our PCR is similar to that obtained with the available commercial kits.

**Keywords:** Tuberculous meningitis, polymerase chain reaction, acquired immunodeficiency syndrome.

**RESUMO**

Meningite é uma forma grave e potencialmente fatal de tuberculose. O diagnóstico envolve a detecção de bacilos álcool-ácido resistentes no líquido cefalorraquidiano por microscopia ou cultura. Entretanto, a dificuldade de detectar o organismo representa um desafio ao diagnóstico. O uso da reação em cadeia da polimerase (PCR) na abordagem diagnóstica de meningite causada por *Mycobacterium tuberculosis* (MTB) tem sido relatado como um método rápido e preciso, com diversos kits comerciais disponíveis. Como alternativa, algumas instituições vêm desenvolvendo testes *in house* com baixo custo. Em nossa instituição, usamos PCR *in house* para tuberculose. O desempenho de nossa PCR para o diagnóstico de meningite causada por MTB foi analisado em 148 pacientes consecutivos, usando a cultura do MBT como padrão-ouro. A sensibilidade da PCR no líquido cefalorraquidiano para o diagnóstico de meningite causada por MTB foi de 50%, especificidade de 98,6% e concordância com cultura de 96% (kappa = 0,52). O desempenho de nossa PCR é semelhante ao obtido com os kits comerciais disponíveis.

**Unitermos:** Meningite tuberculosa, reação em cadeia da polimerase, síndrome da imunodeficiência adquirida.

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Tuberculosis is a public-health problem of global importance. According to the World Health Organization (WHO), more than 8 million people develop tuberculosis each year, with 2 million deaths (1). With the epidemic of human immunodeficiency virus (HIV) infection, disseminated and extrapulmonary forms are increasingly seen. Extrapulmonary involvement is found in more than half of acquired immunodeficiency syndrome (AIDS) patients, and the risk of disseminated disease increases with advancing immunosuppression (2).

Tuberculous meningitis is a potentially fatal form of tuberculosis, with serious long-term consequences. Confirmation of the diagnosis is challenging. The traditional approach of clinical

data with added biochemical and cytological findings of the cerebrospinal fluid (CSF) cannot confirm or exclude the condition. Typical results include lymphocytic pleocytosis, elevated protein and low glucose contents. The diagnostic workup also involves detection of acid-fast bacilli (AFB) in the CSF by microscopy and culture. Due to the paucibacillary characteristic of this condition, sensitivity of these tests is low (3,4). Collecting large amounts of CSF (30 mL) may provide better diagnostic results (4). Some studies indicate that an elevated CSF adenosine deaminase (ADA) level may support the diagnosis in a proper clinical setting (5), but the cut-off value is not clear and additional studies are necessary.

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Use of polymerase chain reaction (PCR) to amplify specific regions of *Mycobacterium tuberculosis* (MTB) genome emerged as a tool for rapid and accurate diagnosis of tuberculous meningitis. The performance of this test has been studied since 1990, with results disclosing high specificity and variable sensitivity (6-8). The exact role of this test in the diagnostic approach remains controversial. Its clinical usefulness appears to be in smear-negative patients, which occurs in the majority of patients with tuberculous meningitis (3).

There are several commercial MTB PCR kits available in the market. Some institutions, most of them in developing countries, have been designing in-house assays, as an inexpensive alternative. A recently published meta-analysis compared the accuracy of commercial and in-house kits for tuberculous meningitis (3). Sensitivity was of 56 and 76%, and specificity was 98 and 92%, for commercial and in-house kits, respectively. Besides the high heterogeneity among studies included, the authors concluded that there is no significant difference between commercial and in-house assays.

Given that the results of the different in-house MTB PCR assays are heterogeneous, it is not possible to extrapolate data from one institution to another. In our institution, a tertiary teaching hospital, we use an in-house assay that has not been previously validated.

A study was conducted to analyze the accuracy of our PCR to diagnose tuberculous meningitis.

## METHODS

### Design

A cross-sectional study was conducted between January 2005 and May 2006 at Hospital de Clínicas de Porto Alegre, located in southern Brazil.

### Patients

Inclusion criteria were all patients with suspected tuberculous meningitis, in which CSF MTB PCR and MTB cultures were ordered. For patients with more than one MTB PCR ordered, only the results on the first sample were considered in the analysis.

### Polymerase chain reaction for *M. tuberculosis* detection

An aliquot of 140 µL of CSF, previously treated with proteinase K (Invitrogen®, São Paulo, Brazil), was used for DNA extraction. The DNA samples were initially submitted to a nested PCR with primers to 65-KDa antigen, common to several

pathogenic mycobacteria. The first nested PCR was performed with the external primers 5' GCC AAG AAG ACC GAT GAC GT 3' and 5' TCG TTG CCC ACC TTG TCC AT 3' that amplify a DNA fragment of 280 base pairs (bp). Ten microliters of CSF DNA were amplified in a 40 µL reaction mixture containing 1.25 U of Taq polymerase (Super-Therm, JMR Holdings, London, United Kingdom), 1.5 mM of MgCl<sub>2</sub> buffer, 250 µM of deoxynucleoside triphosphates (ABgene®, Epsom, UK), 100 nM of external "primers" (Invitrogen®, Carlsbad, USA) and distilled water, totalizing 50 µL. The mixture was submitted to a denaturation period of 100 s at 94 °C, followed by 33 cycles of amplification (each cycle consisted of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s).

In the second reaction, the amplicon of the first DNA reaction was amplified to a DNA fragment of 142 bp, using the internal primers 5' CCA ACC CGC TCG GTC TCA A 3' and 5' CCG ATG GAC TGG TCA CCC 3'. Two microliters of the amplicon from the first reaction were amplified in a 24 µL reaction mixture containing 1.25 U of Taq polymerase (Super-Therm, JMR Holdings, London, United Kingdom), 1.5 mM of MgCl<sub>2</sub> buffer, 250 µM of deoxynucleoside triphosphates (ABgene®, Epsom, UK), 100 nM of internal primers (Invitrogen®, Carlsbad, USA) and distilled water, totalizing 26 µL. The mixture was submitted to a denaturation period of 45 s at 94 °C, followed by 33 cycles of amplification (each cycle consisted of 94 °C for 20 s, 55 °C for 20 s and 72 °C for 30 s).

In those samples which *Mycobacterium sp.* DNA was amplified, a second nested PCR was performed. For the amplification of MBT complex DNA, external primers TB290 5' GGC GGG ACA ACG CCG AAT TGC GAA 3' and TB856 5' CGA GCG TAG GCG TCG GTG ACA AAG 3' were used to generate a fragment of 600 bp. Ten microliters of CSF DNA were amplified in a 40 µL reaction mixture containing 1.25 U of Taq polymerase (Super-Therm, JMR Holdings, London, United Kingdom), 1.5 mM of MgCl<sub>2</sub> buffer, 250 µM of deoxynucleoside triphosphates (ABgene®, Epsom, UK), 500 nM of external primers (Invitrogen®, Carlsbad, USA) and distilled water, totalizing 50 µL. The mixture was submitted to a denaturation period of 100 s at 94 °C, followed by 33 cycles of amplification (each cycle consisted of 94 °C for 30 s, 65 °C for 30 s and 72 °C for 30 s).

In the second reaction, the amplicon of the first DNA reaction was amplified to a DNA fragment of 170 bp, using the internal primers TB500 5' TAC TAC GAC CAG ATC 3' and TB607 5' TTG GTG ATC AGC CGT 3' (9). Two

microliters of the amplicon from the first reaction were amplified in a 24  $\mu$ L reaction mixture containing 1.25 U of Taq polymerase (Super-Therm, JMR Holdings, London, United Kingdom), 1.5 mM of  $MgCl_2$  buffer, 250  $\mu$ M of deoxynucleoside triphosphates (ABgene<sup>®</sup>, Epsom, UK), 500 nM of internal primers (Invitrogen<sup>®</sup>, Carlsbad, USA) and distilled water, totalizing 26  $\mu$ L. The mixture was submitted to a denaturation period of 45 s at 94 °C, followed by 33 cycles of amplification (each cycle consisted of 94 °C for 20 s, 52 °C for 20 s and 72 °C for 30 s).

Negative (distilled water) and positive controls (H37RV culture of *Mycobacterium*) were used for all reactions. Visualization of the amplification products was performed using 10  $\mu$ L of the final volume in 0.5  $\mu$ g/mL ethidium bromide 2% agarose gel electrophoresis under ultraviolet light.

### ***M. tuberculosis* cultures**

All clinical specimens submitted for mycobacterial culture were processed and simultaneously cultured on BACTEC broth medium (Becton Dickinson, Sparks, Md.) and Lowenstein-Jensen (LJ) solid medium. The BACTEC broth medium (12B) contains palmitic acid labeled with <sup>14</sup>C; the mycobacteria catabolize the <sup>14</sup>C-labeled palmitic acid and release <sup>14</sup>CO<sub>2</sub>; the <sup>14</sup>CO<sub>2</sub> in the headspace gas is measured by an automated radiometric system (BACTEC 460 TB). The characteristic mycobacteria colonial morphology was evaluated on LJ and AFB smears were also performed on the sediments. Differentiation of mycobacterial isolates as MTB or *Mycobacterium sp.* other than tuberculosis (MOTT) was based on the BACTEC NAP test.

Positive control standard culture of MBT H37RV was processed with every batch of specimens.

### ***Criteria for diagnosing tuberculous meningitis***

All patient records were revised and a clinical diagnosis of MTB meningitis was considered when the patient had a positive culture for MTB in CSF. An alternative diagnosis was considered present when MTB CSF cultures were negative.

### ***Statistical analysis***

A two-by-two table was performed using the results obtained, with sensitivity, specificity, likelihood ratios and predictive values derived from

it. Intertest agreement was evaluated by concordance and kappa value. Statistical analysis was performed using the software Statistical Package for the Social Sciences 13.0 (SPSS, Inc., Chicago, IL).

### ***Bioethics***

The project was approved by the Ethics Committee of the institution.

## **RESULTS**

A total of 148 patients were included in the analysis. The baseline characteristics of the patients are described in Table 1. Positive CSF cultures were found in 4% and positive CSF PCR in 3.3 % of the patients. Of the five patients with positive CSF PCR, two patients had negative CSF cultures. All patients with positive CSF cultures or PCR were considered to have MTB meningitis and received specific treatment.

The performance of CSF PCR was compared with CSF cultures results (Table 2).

**Table 1. Patients' baseline characteristics**

Age mean (SD)	38 (16)
Male sex n (%)	88 (59.5)
HIV + n (%)	106 (71.6)
Alternative diagnosis n (%)	100 (67)
Cryptococcus meningitis	15 (15)
CNS Toxoplasmosis	14 (14)
Viral meningitis	9 (9)
Neurosyphilis	4 (4)
Bacterial meningitis	4 (4)
Other diagnosis	54 (54)

CNS= central nervous system; HIV= human immunodeficiency syndrome; n= number of patients; SD= standard deviation.

**Table 2. Performance of polymerase chain reaction against culture results**

	<b>MTB culture</b>
<b>Sensitivity</b>	0.50 (0.18-0.81)
<b>Specificity</b>	0.98 (0.95-0.99)
<b>PPV</b>	0.60 (0.23-0.88)
<b>NPV</b>	0.97 (0.94-0.99)
<b>LR+</b>	35.5 (7.2-174.4)
<b>LR-</b>	0.50 (0.22-1.12)
<b>Agreement</b>	0.96 (0.18)
<b>Kappa</b>	0.52 (0.16-0.89)

NPV = negative predictive value; PPV = positive predictive value; LR+ = positive likelihood ratio; LR- = negative likelihood ratio.

\* Data are shown with confidence interval of 95% in brackets, except for agreement, which is shown with standard error.

When data from 47 patients without other diagnoses were analyzed, the high specificity (95.1%; 95% CI 83.9-98.7) and positive likelihood ratio (10.2; 95%CI 2.1-49.3), with similar sensitivity for the *M. tuberculosis* PCR (50%; 95%CI 18.8-81.2), persisted.

## DISCUSSION

In our study, a large number of patients without definitive diagnosis of tuberculous meningitis were included, which reflects the scenario of a tertiary teaching hospital with a great attendance of HIV positive patients that present with multiple CNS syndromes similar to tuberculous meningitis. This can explain the low prevalence of MTB meningitis among those with high clinical suspicion of the condition.

Although anti-tuberculous treatment has been offered to 17% of the patients, the MTB culture positivity was much lower (4%). Some aspects can explain the discrepancy. The paucibacillarity of the condition in CSF with few microorganisms able to grow and previous use of anti-tuberculous drugs lower the sensitivity of this method, considered the gold standard for diagnosis. In addition, presumptive diagnosis can be overestimated. In an endemic region for tuberculosis, clinical suspicious of MTB meningitis, a potentially fatal condition, mandates treatment despite lack of confirmation.

In our institution, even without a clinical validation, MTB PCR has been available as a diagnostic tool since 2000. This is the first study to analyze the performance of our MTB PCR in its real clinical setting. The high specificity found in this study is in agreement with previously reported data (3,6,7). In a meta-analysis published by Pai M. et al. (3), the sensitivity of commercial kits was 56%, similar to that found in the present study. A positive MTB PCR is virtually diagnostic of tuberculous meningitis. Taking into consideration the low sensitivity of MTB culture and the long time required for results, the PCR aggregates in saving time and increasing sensitivity for MTB diagnosis.

We provide a methodologically detailed in-house technique for MTB PCR that can be reproduced by others. In contrast to commercial kits, in-house techniques offer the advantage of lower costs, maintaining similar diagnostic

performance. This is a very important issue in developing countries where tuberculosis is epidemic and few financial resources are available to public health.

In order to improve the validation of the MTB PCR used in our institution, we aim to expand the number of patients and analyze MTB PCR performance in other biological materials.

## REFERENCES

1. World Health Organization (WHO). Global tuberculosis control. WHO Report 2001. Geneva: WHO; 2001. p. 18-9.
2. Jones BE, Young SM, Antoniskis D, Davidson PT, Kramer F, Barnes PF. Relationship of the manifestations of tuberculosis to CD4 cell counts in patients with human immunodeficiency virus infection. *Am Rev Respir Dis.* 1993;148:1292-7.
3. Pai M, Flores LL, Pai N, Hubbard A, Rilley LW, Colford Jr JM. Diagnostic accuracy of nucleic acid amplification test for tuberculous meningitis: a systematic review and meta-analysis. *Lancet Infect Dis.* 2003;3:633-43.
4. Thwaites GE, Tran TH. Tuberculous meningitis: too many questions, too few answers. *Lancet Neurol.* 2005;4:160-70.
5. Ribera E, Martinez-Vazquez JM, Ocana I, Segura RM, Pascual C. Activity of adenosine deaminase in cerebrospinal fluid for the diagnosis and follow-up of tuberculous meningitis in adults. *J Infect Dis.* 1987;155:603-7.
6. Kaneko K, Onodera O, Miyatake T, Tsuji S. Rapid diagnosis of tuberculous meningitis by polymerase chain reaction (PCR). *Neurology.* 1990;40:1617-8.
7. Folgueira L, Delgado R, Palenque E, Noriega AR. Polymerase chain reaction for rapid diagnosis of tuberculous meningitis in AIDS patients. *Neurology.* 1994;44:1336-8.
8. Kox LF, Kuijper S, Kolk AH. Early diagnosis of tuberculous meningitis by polymerase chain reaction. *Neurology.* 1995;45:2228-32.
9. Sechi LA, Pinna MP, Sanna A, et al. Detection of *Mycobacterium tuberculosis* by PCR analysis of urine and other clinical samples from AIDS and non-HIV-infected patients. *Mol Cell Probes.* 1997;11:281-5.
10. Perosio PM, Frank TS. Detection and species identification of mycobacteria in paraffin sections of lung biopsy specimens by polymerase chain reaction. *Am J Clin Pathol.* 1993;100:643-7.

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